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CHARACTERIZATION OF NOVEL GLOMERULAR PROTEINS: ROLE IN PHYSIOLOGY AND DISEASE

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CHARACTERIZATION OF NOVEL GLOMERULAR PROTEINS:
ROLE IN PHYSIOLOGY AND DISEASE
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Maximilian, Wilmer and Minda.

ABSTRACT

Glomerular damage accounts for the majority of chronic kidney disease cases, the prevalence of which is dramatically escalating worldwide, mainly due to increase in diabetes and associated nephropathy. The costs for treatment of CKD represent an enormous burden to the health care system. Chronic kidney disease frequently progresses to end-stage renal disease with the only curative treatment options today are lifelong dialysis or kidney transplantation. In order to better understand and treat glomerular disease before it has reached this irreversible stage, it is imperative to understand the cell biology and physiology of the glomerulus including delineation of the molecular make-up of glomerular filtration barrier. This barrier constitutes the endothelial cells of the glomerular capillaries with its bound surface layer of a carbohydrate-rich meshwork (glycocalyx), the glomerular basement membrane, and the podocytes with the sub podocytes space and the attached slit diaphragm spanning between neighboring and interdigitating foot processes. Even though the concerted interplay of all layers of the glomerular filtration barrier is important for the ultra-filtration of the plasma, the podocytes and their injuries have recently been acknowledged as the major culprit of glomerular disease.

In this thesis work, I describe two novel podocyte specific proteins, TDRD5 and RHPN1, which we show in vivo to be important for the integrity of the glomerular filtration barrier.

TDRD5 has previously been considered a male germ cell line specific protein important for retro transposon silencing and spermiogenesis in mice. Here I show that it is expressed in mouse and human podocytes and is enriched in the zebrafish glomerulus. By using the in vivo morpholino knock down technique in zebrafish larvae it was demonstrated that TDRD5 expression is required for the proper formation of the zebrafish pronephros

RHPN1 is a RHOA binding protein which functions as an attenuator of the polymerization of actin stress fibers by regulating the phosphorylation of nonmuscle myosin II regulatory light chain through RHOA downstream effectors. Deletion of the *Rhpn1* gene leads to neonatal albuminuria and glomerular basement membrane abnormality. The phenotype was characterized.

As a part of efforts to elucidate expression signatures of glomerular diseases, the global glomeruli expression pattern of Adriamycin treated nephrotic mice was analyzed by RNA sequencing. The Adriamycin induced nephropathy mouse model mimics the human disease focal segmental glomerular sclerosis which constitutes one of the main causes of chronic kidney disease.

The diabetic nephropathy associated single nucleotide polymorphism 3q locus was shown in our work to be a remote *cis*-acting variant differentially regulating glomerular NCK1 expression, implicating an important role for glomerular NCK1 in diabetic nephropathy pathogenesis.

LIST OF SCIENTIFIC PAPERS

I. Ann-Charlotte Andersson, Lwaki Ebarasi, Bing He, Mark Lal, Kjell Hultenby, Jaakko Patrakka, Karl Tryggvason.
Podocyte protein Tdrd5 is required for the integrity of the zebrafish pronephros. Manuscript NOV14.

II. Ann-Charlotte Andersson, Jing Guo, Masatoshi Nukui, Jaakko Patrakka, Guillem Genove, Karl Tryggvason, Liqun He.
Global transcriptome profiling of mouse glomeruli in Adriamycin-induced nephritis using RNA-sequencing. Manuscript NOV14.

III. Mark A Lal, Ann-Charlotte Andersson, Kan Katayama, Zhijie Xiao, Masatoshi Nukui, Kjell Hultenby, Annika Wernerson, Karl Tryggvason.
Rhophilin-1 is a key regulator of the podocyte cytoskeleton and is essential for glomerular filtration. *J Am Soc Nephrol* 26: 2014.

IV. Bing He, Anne-May Österholm, Juha Ojala, Ann-Charlotte Andersson, Karl Tryggvason.
A remote cis-acting variant at 3q links glomerular NCK1 to diabetic nephropathy. PLoS One 8: e56414, 2013.

CONTENTS

LIST OF ABBREVIATIONS

ADR	Adriamycin
BUN	Blood Urea Nitrogen
cDNA	Complementary Deoxyribonucleic Acid
CKD	Chronic Kidney Disease
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-Mesenchymal Transition
ESL	Endothelial Surface Layer
ESRD	End Stage Renal Disease
FACS	Fluorescence activated cell sorting
FSGS	Focal Segmental Glomerular Sclerosis
GBM	Glomerular Basement Membrane
GFB	Glomerular Filtration Barrier
GAG	Glycosaminoglycan
HE	Hematoxylin/ Eosin staining
IPS	Inter Podocyte Space
MET	Mesenchymal-to-Epithelial Transition
NM II-RLC	Nonmuscle Myosin II Regulatory Light Chain
PSD	Post Synaptic Density
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SD	Slit Diaphragm
SDS-PAGE	Sodium Dodecyl Sulfate Polyakrylamidelektrofores
SMC	Smooth Muscle Cell
SEP	Sub podocyte space Exit Pore
SPS	Sub Podocyte Space
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

1 INTRODUCTION: REVIEW OF LITERATURE

1.1 KIDNEY GENERAL ANATOMY AND FUNCTION

The kidneys are two bean shaped organs located at the rear of the abdominal cavity. They receive about 20 % of the cardiac output and drain blood from the paired renal arteries and veins, respectively. The kidneys filter small molecules and waste products from the blood and excrete a part of them into the urine, while the rest is reabsorbed from the ultra-filtrate (primary urine) and re-entered into the blood circulation. In this way, the kidneys produce daily about 180 liters of primary filtrate most of which is reabsorbed in the tubules, generating approximately one liter of excreted urine a day. Proper secretion and reabsorption of electrolytes and other compounds ensures that the body's homeostasis is maintained regulating both pH and blood pressure. Plasma proteins of the size of albumin or larger do not pass the kidney filter, which allows important macromolecules such as immunoglobulins and transport proteins to be retained in the blood and also albumin which maintains the osmolality of the plasma. The kidneys are also part of the endocrine system both producing and acting upon hormonal signals (1-3).

1.1.1 Nephron

The nephron (Figure 1b, c), the main functional unit of the kidney, is composed of the renal corpuscle, which is situated at the distal end in the kidney cortex, and tubule, which reaches far into the medulla prior to its connection with the collecting duct system and ureter before spilling out into the urinary bladder. The renal corpuscle constitutes the Bowman's capsule and the glomerular tuft made of capillaries that have branched out of small arterioles (Figure 4). Oxygenated blood enters the kidney trough the renal artery, which is stepwise arborized into smaller arterioles which finally reach the afferent arterioles that enter the Bowman's capsule of the ~1 million nephrons of the human kidney. Small water-soluble waste products and other solutes that are filtered from the blood form the primary urine of the Bowman's space. The large volume of primary urine is then progressively diminished as it is led into the proximal tubule and further through the tubular system while continuously being absorbed by the tubular epithelial cells and transported to the underlying associated capillaries and interstitium. Via the collecting duct and ureter approximately one liter per day enters the bladder that is finally excreted through the urethra.

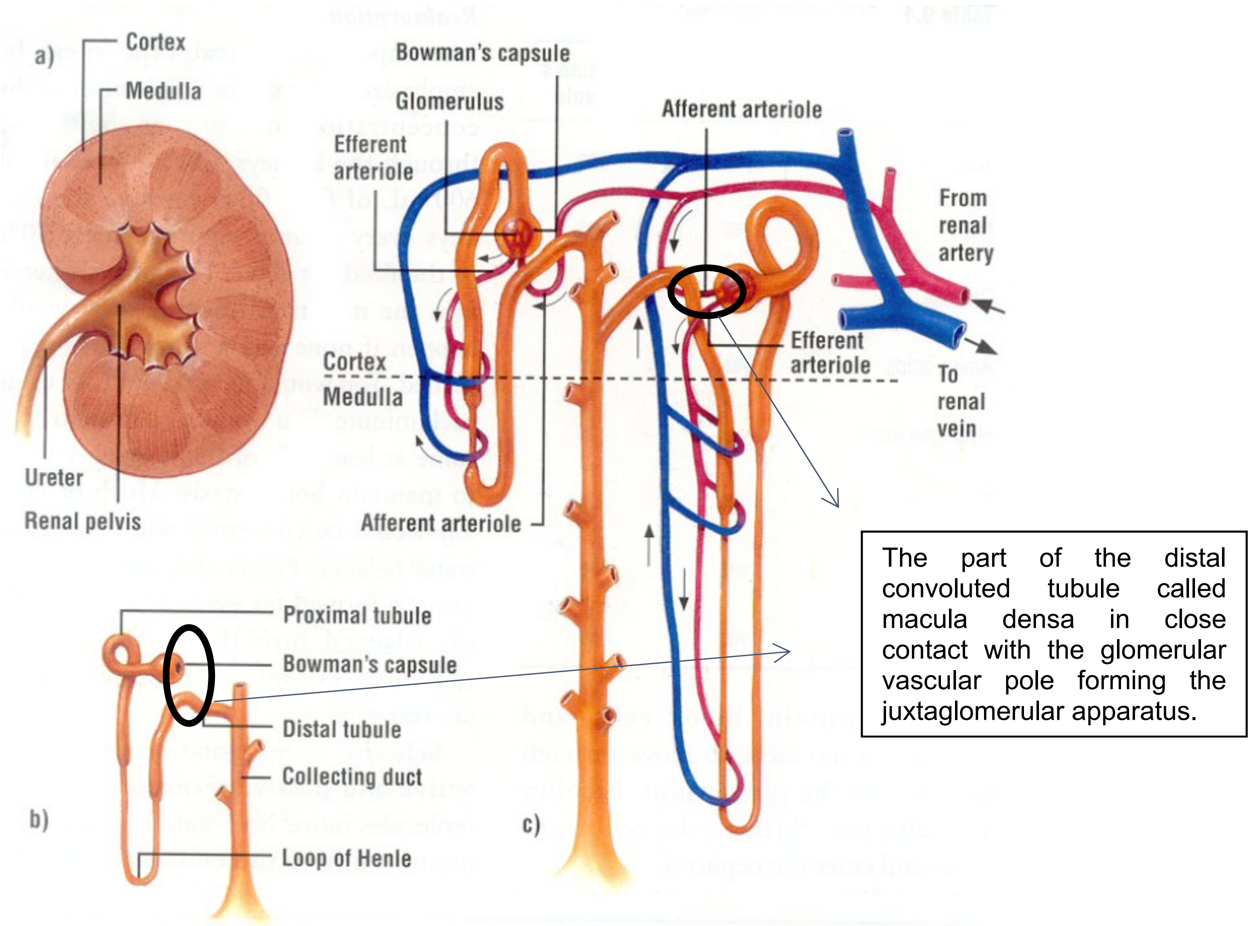


Figure1. The urinary system of the kidney. a) A cross section of the kidney b) Different parts of the nephron. c) The nephron here with the peritubular capillaries which allows for the reabsorption and secretion between blood and the filtrate of the nephron (Reprint cae1.com).

1.1.1.1. Renal corpuscle

The renal corpuscle (Figure 1c) is defined as the Bowman's capsule together with the glomerular capillary tuft formed by branching of the afferent arteriole invading the capsule (Figure 1b, c) (4-6).

1.1.1.2 Proximal convoluted tubule

The epithelial cell layer lining the proximal convoluted tubule (Figure 1b) is connected by tight junctions that prevent waste products in the primary urine from re-entering the blood stream via the interstitial fluid. As approximately 180 liters of primary filtrate is produced daily and the final excreted volume of urine is only about one liter, the need of a highly effective tubular reabsorption mechanism is imperative. Also, apart from waste products, ions and solutes small enough to slip through the glomerular filtration barrier (GFB) are also present in the primary urine and need to be reabsorbed. For this function, the epithelial cell layer is covered with microvilli that increase its surface area and the number of associated active and passive transporters. The proximal tubule reabsorbs 100% of the glucose and amino acids from the glomerular filtrate, and about 70% of the sodium and water. The water is passively transferred into the hypertonic interstitium by osmosis (4-6).

1.1.1.3. Loop of Henle

The loop of Henle (Figure 1c) is the U-shaped portion of the tubule whose primary function is to recover salt and water from the urine. It consists of three different functional and distinguishable portions:

- the descending limb
- the thin followed by the thick ascending limb

The thin descending loop of Henle receives the filtrate from the proximal tubule. As the medulla surrounding the descending limb is hypertonic water leaves the tubule by osmosis. Thus, at the bottom of the loop of Henle the fluid is highly enriched in salt and urea. When the tube, folding back on itself, again heads towards the cortex and becomes the thin ascending loop of Henle salt is passively diffusing into the medulla because of the higher salt concentration in the tubule than in the surrounding tissue. If necessary, even more salt could be passed out into the medulla when the filtrate reaches the thick ascending limb because of the active salt transporters found here, making possible for more water to leave the descending limb by osmosis (4-6).

1.1.1.4. Distal convoluted tubule

In the distal convoluted tubule (Figure 1b, c), Na^{2+} is reabsorbed through the coupled secretion of H^+ and K^+ from the tubular filtrate, which contributes to the regulation of the acid-base balance. The distal tubule is usually impermeable to water, but in the presence of antidiuretic hormone a further concentration of the urine is made possible. At one point when the tubule loops back into the cortex, a region of the tubule called the macula densa (Figure 1b and c, Figure 3), comes in close contact with the glomerular vascular pole, thus forming the juxtaglomerular apparatus which is important in the control of systemic blood pressure and volume (7). The cells of the macula densa are able to relay the Na^{2+} concentration of the filtrate, a read-out of the systemic blood pressure status to the juxtaglomerular apparatus, which triggers various responses to adjust the pressure within the glomeruli accordingly (4-6).

1.1.1.5. Connecting tubule

In 2009 it was determined by using a *Six2*GFPCre transgenic mouse that the connecting tubule which fuses the nephron tubules and the collecting duct tips is derived from the cap mesenchyme and not as previously hypothesized from the ureteric bud, and should thus be regarded as a part of the nephron (8).

1.1.2 Collecting duct system

Several connecting tubule from adjacent nephrons merge to form cortical collecting tubules which then spill into descending cortical collecting ducts (Figure 1b), further down named the medullary collecting duct before entering the renal pelvis on its way to the ureter. The collecting duct system participates in the regulation of electrolytes and due to the hypertonic medulla also further adds to water absorption (4-6).

1.2 NEPHRON DEVELOPMENT

The renal primordial structures develop in a paired arrangement in the cervical region of the embryo, extending from the 4th to the 14th somite. They arise from the intermediate mesoderm and the development is divided up in three distinguishable stages; the pro-, meso- and meta-nephric stages (9) (Figure 2).

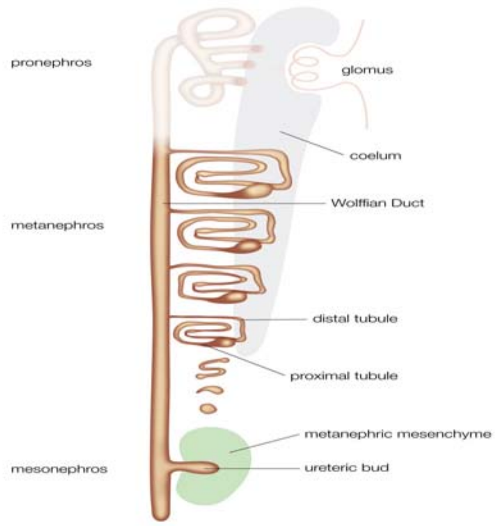


Figure 2. The pro-, meso- and meta-nephric stages of the mammalian kidney development. (Reprint with kind permission from Springer Link: Moritz et al.:Factors influencing mammalian kidney development: implications for health in adult life. Advances in anatomy, embryology, and cell biology 196: 1-78, 2008)

1.2.1 Pro-nephric stage

The pronephric stage appears in human at 3 weeks of gestational age (10). In mammals, it is located in a vestigial structure and consists of a set of paired tubules (the nephrotomes) spilling into a pair of primary ducts, which extends into the cloaca. The pronephros has no filtering or osmoregulatory function but is important for the induction of the next developmental stage, the mesoneophros (2) (Figure 2).

1.2.2 Meso-nephric stage

The disappearance of the pronephros and the formation of the mesoneophros take place around the 5th week of human gestation (10). As the pronephric ducts extends caudally in the embryo it induces the nearby intermediate mesoderm to develop into mesonephric tubules. Each of the structures will get in contact with a tuft of the branching aorta. The tubules then encapsulate the capillaries and filtration of the blood is initiated. The filtrate is led through the tubules and drained into the continuation of the pronepric duct, now named the mesonephric or the Wolffian duct (2) (Figure 2).

1.2.3 Meta-nephric stage

During the 6th week of human gestation the ureteric bud grows out from the mesonephric duct in the proximity to its attachment to the cloaca (10). Reciprocal interplay between the ureteric bud and the metanephric mesenchyme will induce the condensation of the metanephric mesenchyme leading to formation of the renal vesicle thus representing a mesenchymal to epithelial transition event. PAX-2 has been shown using gene knock-out technique to be essential for this conversion of cells of the metanephric mesenchyme to cells of the renal vesicle as in its absence no formation of the vesicle occurs (11). The cells of the renal vesicle are further divided up into two separate populations defined by their expression of FOXD1 and SIX1 respectively. FOXD1 positive progenitor population will give rise to diverse cell types such as mesangial cells or angioblasts, whereas all the epithelial cell types of the nephron originate from the SIX1 positive cells (12).The cells of the vesicle are simple, polygonal vividly multiplying and connected apically by tight junction proteins such as ZO-1 (13) but also desmosomal proteins (14). The renal vesicle which represents the earliest epithelial structure of the nephron then progresses through the comma, S-shaped and capillary stages of nephrogenesis (15) (Figure 3). The metanephric mesenchyme will in this manner contribute to the formation of the nephron while the ureteric bud constitutes the origin of the collecting ducts, renal calyces and urinary tract collection system (16). During the comma and S-shaped stages angioblasts within the metanephric mesenchyme starts to interact with the cell clusters and through paracrine signalling VEGF secreted from the non-matured podocytes attracts endothelial cell progenitors to migrate into the S-shaped body cleft (17, 18). The process of vasculogenesis matures further during the capillary stage. Now ZO-1 relocates from its apical to a basal region with the concomitant disappearance of desmosomal proteins (14, 19) and the presumptive podocytes start to establish their characteristic complex cell architecture with formation of foot processes and expression of slit diaphragm proteins such as nephrin (19), CD2AP (20) and podocin (21). Around this stage TDRD5 is also expressed constituting one of the anchor genes of the visceral epithelial cells at Theiler stage23 (15day dpc in mouse corresponding to day53 in human gestation) (22, 23). The process is finalized when the Bowman’s capsule encompasses the capillary tuft, together forming the renal corpuscle. WT-1 remains a podocyte specific marker throughout ontogeny and adulthood (24, 25), and dominant mutation of the encoding gene are associated with the Denys-Drash and Frasier syndromes (26, 27). At 36 weeks of human gestation nephrogenesis is complete and each kidney has a full set of nephrons.

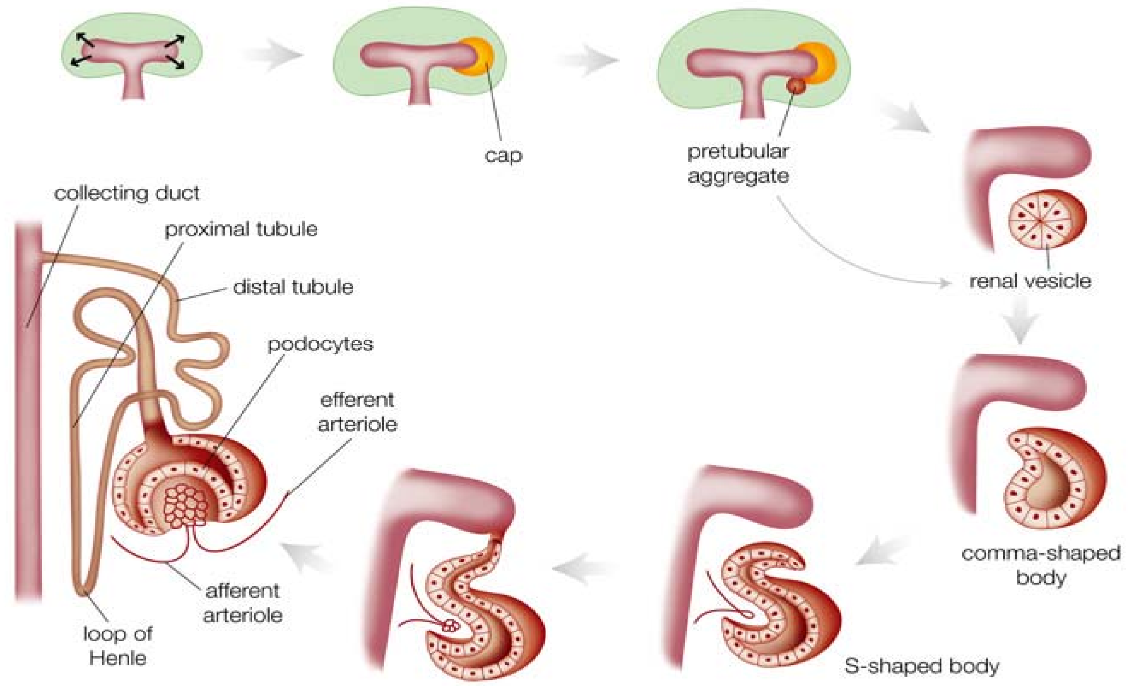
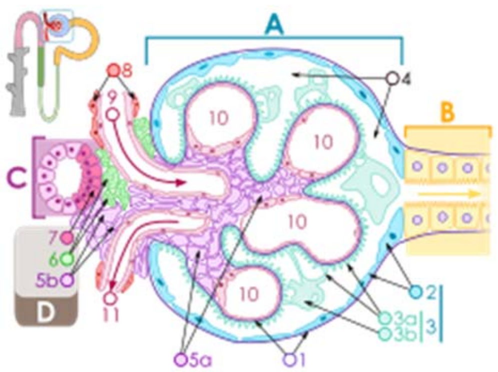


Figure 3. The progression through the meta-nephric stage of mammalian renal development. (Reprint with kind permission from Springer Link; Moritz et al.; Factors influencing mammalian kidney development: implications for health in adult life. Advances in anatomy, embryology, and cell biology 196: 1-78, 2008).

1.3 RENAL CORPUSCLE

The renal corpuscle is made up of the part of the nephron named the Bowman's capsule and the glomeruli which is the capillary tuft it is invaded by.



A	Renal corpuscle
B	Proximal tubule
C	Distal convoluted tubule
D	Juxtaglomerular apparatus
1	Basement membrane (basal lamina)
2	Bowman's capsule (parietal cell layer)
3	Bowman's capsule (visceral layer)
3a-b	Foot processes (pedicels)
4	Bowman's space (urinary space)
5a	Mesangium (Intra glomerular cells)
5b	Mesangium (extra glomerular cells)
6	Granular cells (juxtaglomerular cells)
7	Macula densa
8	Myocytes (smooth muscle)
9	Afferent arteriole
10	Glomerular capillary
11	Efferent arteriole

Figure 4. The constituents of the renal corpuscle.

1.3.1 Bowman's capsule

The Bowman's capsule is lined by a 0,1-0,3 μM thick simple squamous epithelium made up of the fourth cell type of the renal corpuscle, the parietal epithelial cells (28). The space enclosed by the parietal cell layer and the visceral epithelial cell layer (podocytes) is where the primary urine, also called the ultra-filtrate, is collected before being led out to the proximal tubule (29). The parietal cells that originate from the same SIX1 positive progenitor cells as the podocytes in the vesicle stage, diversify from the podocyte progenitor cells at the S-shaped stage (30). Transitional cells in the intersection between parietal cell and the podocytes have been seen in glomeruli which each express both parietal and visceral epithelial cell markers and are able to form foot processes onto Bowman's basement membrane (31, 32). Others have proposed that a pool of the parietal cell population can, under some circumstances, migrate onto the vascular tuft and differentiate into podocytes (33, 34). These findings are interesting considering that the podocytes are terminally differentiated and unable to replenish in case of loss due to damage or senescence. However, some investigations have shown this kind of repair as being maladaptive in itself causing albuminuria and glomerulosclerosis (35).

1.3.2 Glomerulus

A technique where glomeruli are transplanted into the anterior chamber of the mouse eye allowing for glomeruli in vivo imaging was recently developed (36). This and other techniques that allows for the study of the glomerulus in an in vivo setting will add greatly to our understanding of this structure which is situated in the Bowman's capsule and contains a capillary tuft of five to eight trunks each subdivided further into 20-40 capillary loops (37). After circulating through the tuft, the blood then exits through the efferent arteriole. A hydrostatic pressure is generated within the glomerulus (~60mmHg) due to its interposition between the afferent and efferent arteriole (38). However, due to the hydrostatic pressure in Bowman's space of ∼20 mmHg (39) plus the afferent colloid osmotic pressure of 25-35 mmHg, the effective filtration pressure is estimated to be ∼10 mmHg (40). The pressure may be varied by hormonal or neuronal cues that regulate the tonus of the smooth muscle properties of both podocytes and mesangial cells which are positioned on the blood and urinary side of the GBM, respectively (41). In this way the ultrafiltration of metabolic waste products and other small solutes such as water, glucose, amino acids, urea and sodium chloride is accomplished, while larger molecules such as albumin, immunoglobulins and plasma transport proteins are retained in the blood flow. As a measure of the permselectivity of the filter the sieving coefficient (Ɵ) is used, defined as the concentration of solute in Bowman's space divided by concentration in the plasma (42). The filtrate represents about 20% of the total plasma volume that enters the glomeruli at any given time. It passes through the fenestrated capillary wall, the basement membrane and the podocyte slit diaphragm before collected in the urinary space of the Bowman's capsule (1, 29, 43-45).

1.4 GLOMERULAR FILTRATION BARRIER

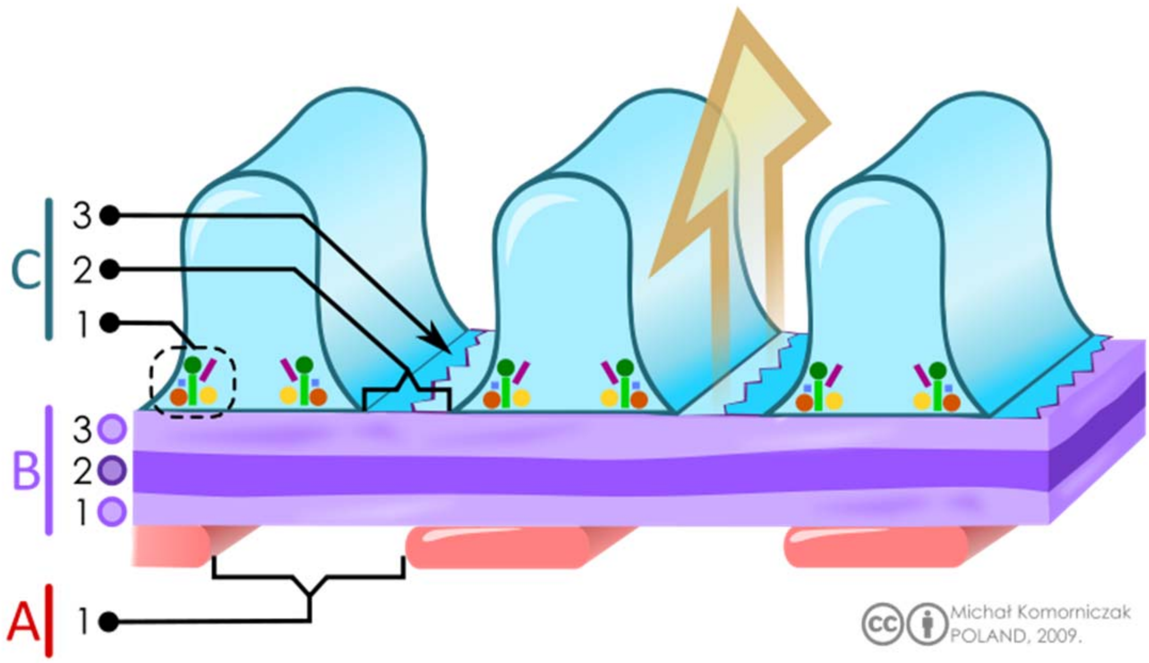


Figure 5. The glomerular filtration barrier, A. Endothelial cells. . Fenestrae. B. Glomerular basement membrane: 1. Lamina rara interna 2. Lamina densa 3. Lamina rara externa C. Podocytes: 1. Slit diaphragm associated proteins. 2. Filtration slit. 3. Diaphragm (Reprint from Wikipedia).

The glomerular filtration barrier (GFB) is considered as a size and charge selective molecular sieve between the blood flowing through the glomerular capillaries and the ultra-filtrate in the urinary space of the Bowman's capsule (46), even though its charge selective properties has been debated (47). Whichever, it has the ability to filter massive amounts of water and small solutes into the Bowman's capsule, while retaining at least 99,9% of albumin and other large proteins in the plasma (48). The classical description of the barrier composition is that of a three layers; the fenestrated endothelium of the capillaries, the superjacent glomerular basement membrane and finally the podocytes with the slit diaphragm spanning in-between the interdigitating foot processes (42). Additionally, an endothelial surface layer, referred to as glycocalyx (49-51), and the so called sub-podocyte space (40, 52) may be added to the list. It is apparent that these layers form a functioning entity that is dependent on the biochemistry, biophysics and cell biology of all its constituents (42, 45, 53). It has been shown that both genetic defects and/or acquired damage to any one layer may lead to proteinuria (54-56), and the course taken in the progression of disease is highly variable and dependent on the etiology of the primary insult (57).

1.4.1 Endothelium

Gene manipulation techniques targeting endothelial cells have generally been global, and thus not allowed specific targeting of glomerular endothelial cells. This has hampered studies on the biology of glomerular endothelial cells, as opposed to the podocytes and mesangial cells. It is however evident that the endothelium is a functional part of the GFB even though 20-50% of the glomerular capillary endothelial surface is occupied by 60-80nm in diameter wide trans cellular fenestrations (58). The large fenestrae are a prerequisite for the high permeability of fluid across the capillary wall, which is an order of magnitude higher than in any other systemic capillary beds (59). The size of the fenestrae has previously diminished discussions the endothelium playing a functional part of the GFB as the openings are ~15x that of a macromolecule such as albumin (42). However, several studies have shown that the endothelium does play an important part in the perm-selective properties of the GFB (56, 60, 61). For example, studies on the VEGF/VEGFR-2 signaling pathway between podocytes and glomerular endothelial cells have strongly supported that notion. Thus, it has been shown that albuminuria may be present in the case of a damaged endothelium without concomitant GBM or podocyte disturbance (62). Other investigators have also suggested that the level of proteinuria does not correlate with podocyte foot process effacement (63). The recent identification of a highly glomerular endothelium specific protein EHD3 (64, 65) may provide new means to specifically target the glomerular endothelial cells in animals.

1.4.2 Endothelial surface layer

Using improved three dimensional electron microscopy techniques, investigators have generated images of a negatively charged glycocalyx bound to the luminal aspect of the endothelium covering and extending into the fenestrae (66, 67). It consists of covalently bound proteoglycans, glycoproteins and sialic acids produced by the endothelial cells that make up the core of this lattice, which in turn is associated with the long linear glycosaminoglycans of heparan sulfates and chondroitin sulfates. To this material circulating plasma molecules such as albumin, orosomucoids and lumican are adsorbed. Together, these components make up a 200-400 nm deep endothelial surface layer (ESL) which may restrict passage of macromolecules based on both charge and steric hindrance (68, 69). Several in vivo and in vitro studies have been made on the role of the ESL as part of the permselective GFB barrier (70). It is surmised that damage of the ESL is a common denominator of diseases of the extrarenal vascular system and such diseases and albuminuria often correlate to each other (71). It is a well-established fact that although diabetes is the most common cause of ESRD, most such diabetic patients also suffer from other forms of microangiopathies and macrovascular as well as cardiovascular complications before reaching this stage. Having identified a protein specifically expressed in glomerular endothelial cells (64) may enable conditional knockouts of genes expressed by those cells.

1.4.3 Glomerular basement membrane

The glomerular basement membrane (GBM) is a 250-400 nm thick specialized sheet-like extracellular matrix the components of which are produced and laid down by the adjacent endothelium and podocyte cells. Transmission electron microscopy of the GBM reveals a three laminar layer with the *lamina rara interna* and *lamina rara externa* facing the endothelium and podocytes, respectively, and an electron dense central layer, the *lamina densa* (72) (Figure 5B). The GBM has been proposed to contribute to the size and charge selective properties of the GFB (73-77), but it also constitutes the main structural support of the glomerular capillary wall. Harboring ligands for membrane proteins of podocytes, endothelium and mesangial cells, being in direct contact with each one of them makes it important also for the paracrine cell to cell signaling system of the different GFB constituents (78, 79). The GBM consists mainly of the macromolecules type IV collagen (80, 81), laminins (80, 82, 83), nidogen (84), and the heparan sulfate proteoglycans such as perlecan and agrin (85), which together produce an interwoven meshwork believed to impart the perm-selective properties to the GBM.

Type IV collagen, the main structural component, is a trimer of α1 and α2 chains (α1α1α2) in the embryo which is replaced after birth by α3α4α5 trimers (86). Mutations in any of the genes for the a3, a4 or a5 chains lead to distortion of the GBM structure and Alport syndrome which is characterize by hematuria and progressive glomerular disease that usually requires dialysis or transplantation treatments (87-89).

Laminins are large heterotrimeric glycoproteins made up of homologous α, β, and γ chains (90). The laminin LN-511 (α5β1γ1) is the only GBM isoform in embryo, but it is replaced during GBM maturation by LN-521 (α5β2γ1) (91). Mutations in the *LAMB2* gene lead to one form of congenital nephrotic syndrome referred to as Pierson syndrome (92, 93). It is speculated that the developmental switch between the laminin and collagen isoforms occur in order to make the mature GBM more resilient or aid the podocytes in their differentiation (94). The glomerular diseases caused by type IV collagen and laminin gene mutations represent direct evidence that the GBM plays a crucial role in the glomerular filtration barrier.

Heparan sulfate proteoglycans (HSPG) contain a core protein with attached heparan sulfate (HS) chains. The HS moieties are negatively charged due to their repeats of sulfated disaccharides (sulfated glycosaminoglycan-GAGs) making up chains of unbranched polysaccharides. Basement membranes in general, and the GBM in particular, have a net anionic charge and HSPGs of the GBM such as perlecan, agrin (95) and collagen XVIII (96) are believed contribute to this negative charge. Perlecan, synthesized by the endothelium (97) which seems to be the most common HSPG in basement membranes in general, is less abundant in the GBM than agrin which is primarily produced by the podocytes (98). Since the work by Brenner and colleagues where they used differently charged dextrans to test to what extent they were able to cross the glomerular filtration barrier (99, 100) and the isolation of heparan sulfates from glomerular basement membranes by Farquhar and colleagues (101) it has been held in belief that these HSPGs play an important role as providers of the GBM charge-selective properties. However, a mouse podocyte- specific deletion of agrin did not lead to any structural or functional defects of the glomerulus even though the negative charge of the GBM was greatly reduced (102). By targeting the perlecan encoding gene *HSPG2* so that the attachment sites for the three HS chains were ablated did not have an effect on the filtering capacity of the mouse kidney, or the ultrastructure of the GBM (103). Furthermore, overall glomerular architecture and renal function was found normal in perlecan-HS/agrin double knock out mice (95) suggesting that the proposed GBM negative charge is not so important as previously thought.

1.4.4 Podocyte

Multiphoton microscopy together with transgenic mice that express multicolor fluorescent proteins in the podocytes now allows for in vivo imaging of this cell (104) which is of a terminally differentiated, highly polarized and arborized parietal epithelial type located on top of the outer surface of the GBM (105). The size of a typical mouse podocyte with a main cell body area of ~50 μm^2, and the length of the primary and secondary foot processes in the several µm and µm range respectively depict an extraordinary type of cell with extraordinary needs (106).

1.4.4.1 General structure

From the voluminous cell body floating aloft in the urinary space extend several primary and secondary processes which extensions, referred to as foot processes attached to the outer surface of the GBM (106). The foot processes (also termed pedicels), interact in an interdigitating fashion with foot processes of a neighboring podocyte. The podocytes are highly polarized cells with basal and apical membrane surfaces which are well defined by the baso-laterally inserted slit diaphragm (SD) (105). Microtubules and intermediate filaments like vimentin and desmin span longitudinally the major processes, whereas contractile actin filaments are critical components of the foot processes (107). The foot process actin filaments, interconnected by α-actinin- 4 (108) are linked via proteins like CD2AP (109), NCK1 (110, 111) and podocin (112, 113) to components of the SD such as nephrin and NEPH1. The podocyte cell body is densely packed with organelles where the nucleus is prominent and endoplasmic reticulum, Golgi and mitochondria are well developed and abundant, witnessing of a high metabolic activity (105, 114).

1.4.4.2 Function

The podocytes are an archetypal example of a cell type with a morphology uniquely tailored to fit its highly specialized functions. Its shape is dependent on the dynamics of the underlying cytoskeleton, which regulation seems to be focused around the slit diaphragm (115, 116). The foot processes with their structure of filamentous actin bundles and subcortical actin meshwork provides anchor-points for the SD, as well as providing the framework for the propagation of outside in signaling cues from SD proteins to the endothelial cells (indirectly through the GBM) and directly to the mesangial cells (at the intersection between the capillary and the mesangium) (110, 111, 117-119). Together with the endothelial cells they also synthesize the GBM components of the heterotrimeric laminin-1 (α1, β1, and γ1 chains) and laminin-11 (α5, β2, and γ1 chains) throughout glomerular development (91), as well as the collagen α3 α4 α5 (IV) network (81). They induce vasculogenesis and fenestrae formation of endothelial cells through the secretion of the pro-angiogenic factors vascular endothelial growth factor (VEGF)-A and angiopoetin-1 (120-122). They have been found to endocytose filtered albumin (123) and immunoglobulins (124). The mesangial cells have by tradition been regarded as the pericytes of the glomerulus referring to their smooth muscle activity in modifying glomerular filtration in response to vasoactive agents. However, podocytes have lately been shown to contract in vivo in response to elevated intracellular [$Ca2^+$] leading to a reduction in capillary diameter (125-127). A study that showed differentiated podocytes to possess contractile properties in vitro also localized the smooth muscle markers smoothelin and calponin and the specific transcription factor myocardin to differentiated podocytes both in vivo and in vitro (128). Due to shared morphological traits (105, 129) and molecular make up (23, 64, 130-132) the podocytes are often compared to the neurons (133). As an ultimate manifestation of the podocytes neuronal properties they have been found to communicate intercellularly through synaptic transmission (134-136). To prevent the GFB from clogging the podocytes have also been hypothesized to take part in its back-washing, by means of the sub podocyte space (40, 68, 69, 137).

1.4.5 Sub podocyte space

The sub podocyte space was first described in 1950 by Gautier and colleagues (138) but as the further investigation of the structure required three dimensional reconstruction of fragile, sub-microscopic (<1μm) formations, it was not until the beginning of the millennium that it could be more closely described using serial section transmission electron microscopy (137), and later also described under physiological relevant conditions (40). Using Serial Block Face Scanning Electron Microscopy, it was found that 50-60% of the GFB was covered by the SPS in rat glomeruli (69). The SPS is defined as *"a space on the urinary side of the GBM that is bound by the GBM and the foot processes on one side and the underside of the podocyte cell body, process, or membrane on the other*" (137). From the SPS the filtrate is passed out through Sub-Podocyte space Exit pores (SPE) that drains into the Inter Podocyte Space (IPS) which is a narrow space in-between the podocyte cell bodies at the center of the glomerulus (137, 139). The filtrate is then passed out through this labyrinthine system out to the periphery of the glomeruli where it drains into the classical Bowman's space. Calculations have shown that the SPS together with a regulated SPE circumference is able to confer resistance to the flux of glomerular filtrate similar to or even greater than the resistance applied by the underlying GFB (40, 137). The physiological significance remains to be elucidated but its speculated that the podocyte in this way is able to sense the rate of filtration which it then reacts upon. It may also serve in backwashing the GFB to prevent its clogging or as a mean of modifying the primary filtrate (40, 68, 69, 137).

1.4.6 Slit diaphragm (see 1.5)

1.5 SLIT DIAPHRAGM

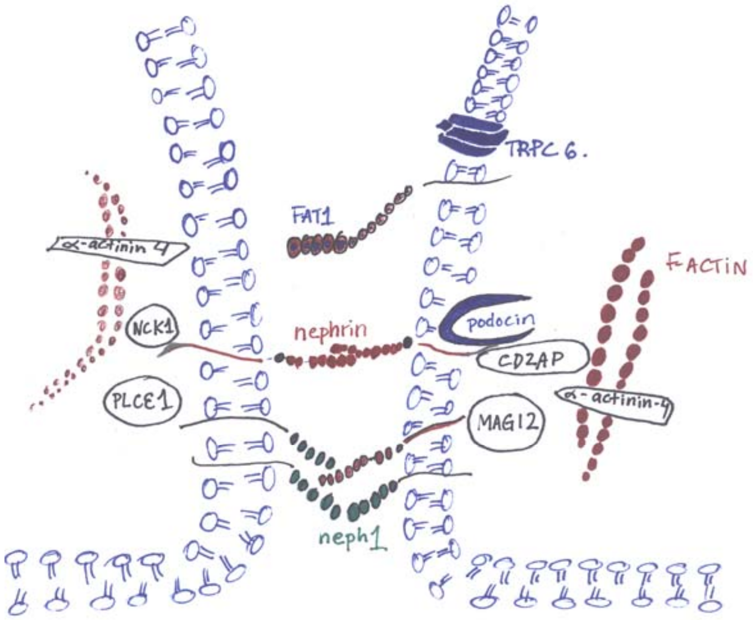


Figure 6. A sketch of the slit diaphragm (SD) with a selection of SD- and SD- associated proteins.

The first description of the slit diaphragm as imaged by Rodewald and Karnovsky in 1974 using electron microscopy on mouse and rat kidney tissue was that of a iso-porous zipper-like substructure, spanning the ~40nm wide slit between neighboring foot processes (140). It was seen to have alternating, periodic cross bridges extending from the baso lateral podocyte plasma membranes to a central filament running parallel to and at equal distances to the cell membrane. The physiological data indicated that the diaphragm would hinder the filtration of proteins at the size of serum albumin an larger (140). This physical description of the diaphragm has however been questioned with the advent of improved imaging techniques with one of the studies instead propose it to be of a heteroporous composition (141). The inter cellular part of the slit diaphragm is believed to consists of nephrin and NEPH1 which belongs together with the highly related NEPH2-3 to the NEPH1-related family of proteins (142). Their extracellular domains are proposed to form homo- and hetero-oligomeric receptor complexes associating via *cis*- and *trans*- interactions (142-145). The transmembrane portions of nephrin and NEPH1 spans the plasmalemma at sites of cholesterol rich micro domains where podocin also resides, a protein which is believed to be necessary for the recruitment of nephrin and NEPH1 as well as other slit membrane associated proteins to these lipid rafts for the assembly of the slit diaphragm complex (146, 147). This slit diaphragm nephrin-NEPH1-podocin complex in turn assembles a specialized signaling protein cluster of various phosphorylation/ adaptor/ effector proteins for the transmission of outside-in information to the actin cytoskeleton (111, 118, 142, 148-152).

Below follow a description of SD- or SD associated proteins that through forward genetics of human renal disease or through genetic targeting in animal models (reverse genetics) has shown to be crucial for SD formation or SD- signaling to the actin cytoskeleton of the podocytes.

1.5.1 Nephrin

The cloning of nephrin (153) and its subsequent localization to the slit diaphragm was a milestone in glomerular research (154, 155). *NPHS1*, the gene encoding nephrin is mutated in the autosomal-recessive disease congenital nephrotic syndrome of the Finnish type (153). The disease is mainly found in the Finnish population with an incidence of ~1 in 10,000 newborns (156), and arises due to the mutated or truncated *NPHS1* protein product leading to massive nonselective proteinuria already in utero (153). Nephrin is an 180kDa type-1 transmembrane glycoprotein of the immunoglobulin superfamily with eight Ig-like domains and a fibronectin type III (FNIII) domain extra cellularly, a transmembrane part, and a small intracellular C-termini (153). Two nephrin molecules are believed to assemble through homophilic head-to-head interactions across the slit forming a zipper like arrangement (154), much like the structure that was described by Rodewald and Karnovsky (140).

It has been shown that nephrin signaling is dependent on interaction with podocin located in lipid rafts (157), and nephrin localization to the lipid rafts is disrupted in mutations in *NPHS2*, the podocin encoding gene (146). The extracellular domain of nephrin also associates with NEPH1 in both *cis*- and *trans*-interactions (143-145). Nephrin signaling is orchestrated through the phosphorylation of crucial tyrosine residues within its cytoplasmic domain by kinases such as FYN (118) and phosphatidyl-insositol-3- kinase (150) and others (111, 148, 149, 151, 152), leading to recruitment of cytoskeletal adaptor proteins such as NCK1/2 via their SH2 domain (110, 111, 119) in turn controlling actin polymerization through N-WASP (158) and the p21 activated kinase serine/threonine kinase PAK (159). Phosphorylation of tyrosine residues in the nephrin C-terminus also recruits the regulatory SH2 domain of PI_3 kinase p85 subunit, in turn, recruiting and activating the PI_3 kinase p110 catalytic subunit (152), which converts the membrane lipid phosphatidylinositol-4, 5-biphosphate (PIP_2) to phosphatidylinositol-3, 4, 5-triphosphate (PIP_3) at the plasma membrane. PIP_3 phospholipids form docking sites for the serine/threonine kinase AKT (160) leading to increased AKT activity and reduced cell death (152). Nephrins involvement in actin regulation is also is depicted by its interaction with CD2-associated protein (CD2AP) (112) which is a protein important for cytoskeletal maintenance and cell morphology (161, 162). Furthermore nephrin binding to IQ motif containing GTPases activating protein (IQGAP) activates the small GTPases RAC1 and CDC42 and their subsequent recruitment of ARP2/3 complex and formin-dependent actin polymerization components (163).

The high conservation of nephrin and NEPH1 through evolution is reflected in their role in the formation of skeletal muscle in drosophila where they drive the myoblast fusion through the interaction between nephrin drosophila homologues hibris and sticks-and-stones on founder cells with NEPH1 homolog dumbfounded on fusion competent cells (164-166). Moreover, in drosophila the process of eye development is strictly regulated by interactions between nephrin homologue hibris expressed on primary pigment cells and NEPH1 homologue roughest on neighboring inter-ommatidial precursor cells. The eyes of hibris and roughest mutant flies get a “rough” appearance as the inter- ommatidial precursor cells are misplaced and form aberrant junctions (167, 168). Another example of nephrin and NEPH1 conserved roles in guiding cells to make appropriate interactions is in C. elegans where nephrin and NEPH1 homologs SYG-2 and SYG-1 respectively are expressed in neighboring heterologous cell types where they make *trans* interaction (169-171). Their expression is essential for guiding motor neurons to form synapses with their appropriate target cells (170, 172, 173)

1.5.2 Podocin

NPHS2 was discovered as a gene mutated in about 20% (174) of patients with early-onset steroid resistant focal segmental glomeruli sclerosis (175). Genetically targeted mice in which podocin was deleted or expressed with missense mutations were present with albuminuria at birth and died within a few weeks (176, 177). Progressive decline in glomerular function and elevated histo- pathological lesions were noted in mice where podocin was deleted in a podocyte specific manner using Cre-loxp technology (178). The *NPHS2* encoded protein podocin which expression is restricted to the podocytes (24, 25, 179) and Sertoli cells of the testis where it is hypothesized to have a role in the blood/testis barrier (180), is a 42kDa integral molecule belonging to the stomatin family of proteins containing the cholesterol binding domain prohibitin (181). It has a hairpin-like structure anchored to the membrane via its central part and with both N- and C-terminus facing the cytosolic side of the slit diaphragm (175). Podocin accumulates as oligos in lipid rafts that are highly defined micro domains dominated by sphingolipids and cholesterol (112, 182). Podocin direct interaction with nephrin is necessary for nephrin signaling (157), and mutations in *Nphs2* disrupts nephrin localization to the lipid rafts (146). Sub- cortically of the rafts an electron-dense, Triton-X resistant material is found, composed of a highly branched actin cytoskeletal network, a region remarkably reminiscent of the Post-Synaptic Density (PSD) of the neurons (113). The C. elegans podocin homologue mechanosensory protein 2 is believed to link the mechanosensory channel and the microtubule cytoskeleton of the touch receptor neuron, and it has been suggested that podocin also acts as such a mechanosensor which enables the podocyte to remodels its cytoskeleton and contract the foot processes as a response to mechanical stimuli through its activation of TRPC6, a $Ca2^+$ channel, thus increasing cytosolic $Ca2^+$ levels regulating actin cytoskeleton polymerization (183-185).

1.5.3 CD2AP

CD2-associated protein (CD2AP) was originally identified as an interaction partner of the mouse T-cell membrane protein CD2 where it is involved in the clustering of CD2 and T-cell polarity (186). It is a 70kDa multidomain scaffolding protein in the kidney primarily expressed in the podocytes (109, 112, 187, 188), where CD2AP directly interacts with the cytoskeletal protein filamentous actin (f-actin) (189, 190) and the actin-bundling protein synaptopodin (191) as well as nephrin (109), podocin (112) and RAC1 (162), thus serving as a linker anchoring slit membrane proteins to the actin cytoskeleton. In human mutations in the CD2AP gene results in FSGS (192-195), and mice deficient in CD2AP develop mesangial sclerosis and die at 6-7 weeks of age due to renal failure (109).

1.5.1 α-actinin-4

The alpha actinins belongs to the spectrin gene superfamily, which represents a diverse group of cytoskeletal proteins (196). The gene *ACTN4* gene encodes a ~100kDa cytosolic nonmuscle, alpha actinin isoform that is necessary for the cross-linking and accurate actin turnover of the podocyte stress fibers (108). Mutations in the human *ACTN4* gene cause an inherited autosomal-dominant form of FSGS (197), and in mice three identified miss sense mutations within exon 8a produced an α -actinin-4 protein with a pathological increased affinity for actin, in which study it was also shown that even though *α-actinin-4* has an ubiquitous expression pattern histological examination of the mice only showed kidney abnormalities (108, 198) highlighting the necessity of tight regulation of the podocyte actin cytoskeleton.

1.5.2 TRPC6

TRPC6 belongs to a family of six human nonselective Ca^{2+} permeable cation channels which are widely expressed in the vertebrate tissue (199). In podocytes it localizes to the foot processes in the vicinity of the SD but also in the cell body and throughout the major processes (200). Nominally functional TRPC channels are tetramers composed of six transmembrane segments with both NH_2 and COOH termini facing the cytosol (199). The activation of TRPC members is believed to be initiated by cascades involving phospholipase C hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) liberating diacylglycerol (DAG) and inositol1,4,5-trisphosphate (IP_3) (201). Several gain of function mutations has been mapped to the cytosolic terminal end of TRPC6 in families with a late onset autosomal dominant form of FSGS (200, 202, 203). Similar to human glomerular disease caused by mutations in *CD2AP* and *ACTN4* respectively the *TRPC6* mutations is not generally present with any other pathological phenotype which is remarkable as all three expressions are widely distributed to other tissues (191, 197, 204, 205). The importance of a tight $Ca2^{+}$ regulation was also highlighted in a study where the up regulation of wild-type TRPC6 was seen in patients suffering from acquired human proteinuric kidney diseases as well as in experimental models of acquired glomerular disease (183). In a study where the relationship between TRPC-mediated calcium entry and cytoskeletal shape and rigidity in podocytes was investigated it was found that calcium influx was mediated by angiotensin II through both TRPC5 and TRPC6 channels. Downstream however it was seen that TRPC5 signaled through RAC1 to promote cell motility, whereas TRPC6 signaled through RHOA to inhibit it (206). The tight antagonistic regulation between RAC1 and RHOA seems as a prerequisite for a normal podocyte phenotype and dysregulation thereof leads to podocytopathy exemplified with the over activation of RHOA in families with gain-of-function mutations in TRPC6 (202, 206). Accordingly mice lacking TRPC6 infused with angiotensin II were significantly less albuminuric than the infused wild- type control (207). In line with our study where the tight regulation by RHPN1 of RHOA dependent stress fiber formation is necessary for healthy podocytes (208) Wang et al. reports that both RHOA activation and inhibition caused albuminuria and foot process effacement (209). Furthermore Zhu et al. reports that inducible activation of RHOA in mice leads to FSGS in a dose dependent manner (210). Thus, compiling data seems to implicate that TRPC6 expression and intracellular calcium levels must be tightly regulated to avoid aberrant RhoGTPase activity.

1.5.3 NCK1/2

NCK1 and NCK2 are two highly related proteins (211) composed of the phosphotyrosine- interacting SH2 and SH3 domains with which they can recruit various other proteins involved in the regulation of actin assembly, such as the NCK1 SH2 domain binding to tyrosine-phosphorylated binding sites of nephrin (110, 111) and its SH3 domains on the other hand binding to neuronal Wiskott-Aldrich syndrome protein (N-WASP) (110, 111), whereby N-WASP activates the ARP2/3 complex and cortactin linking the nephrin-NCK complex to the actin cytoskeleton (115, 161). NCK recruitment to phosphorylated nephrin is initiated during development, injury or repair when cytoskeletal reorganization and rapid actin polymerization is required (111). Mice deficient of both NCK1 and NCK2 in podocytes develop nephrotic range proteinuria, in addition to ultra- structural changes in glomeruli similar to those found in human end-stage renal disease (110).

1.5.4 FAT1

The 500-kDa FAT1, a protein that belongs to the protocadherin superfamily is localized to the slit area where it has been found to co localize with nephrin and ZO-1 (212). Newborn FAT1 null mice display effaced foot processes without slit diaphragms and die within 48 hours (213). It is believed that FAT1 takes part in the generation of the necessary intercellular adhesion framework between foot processes while at the same time maintaining the junctional distance. In support of this is the involvement of FAT1 in actin dynamics (214) and the active re-arrangement of actin cytoskeleton during progression of podocytopathy (215, 216).

1.5.5 MAGI-2

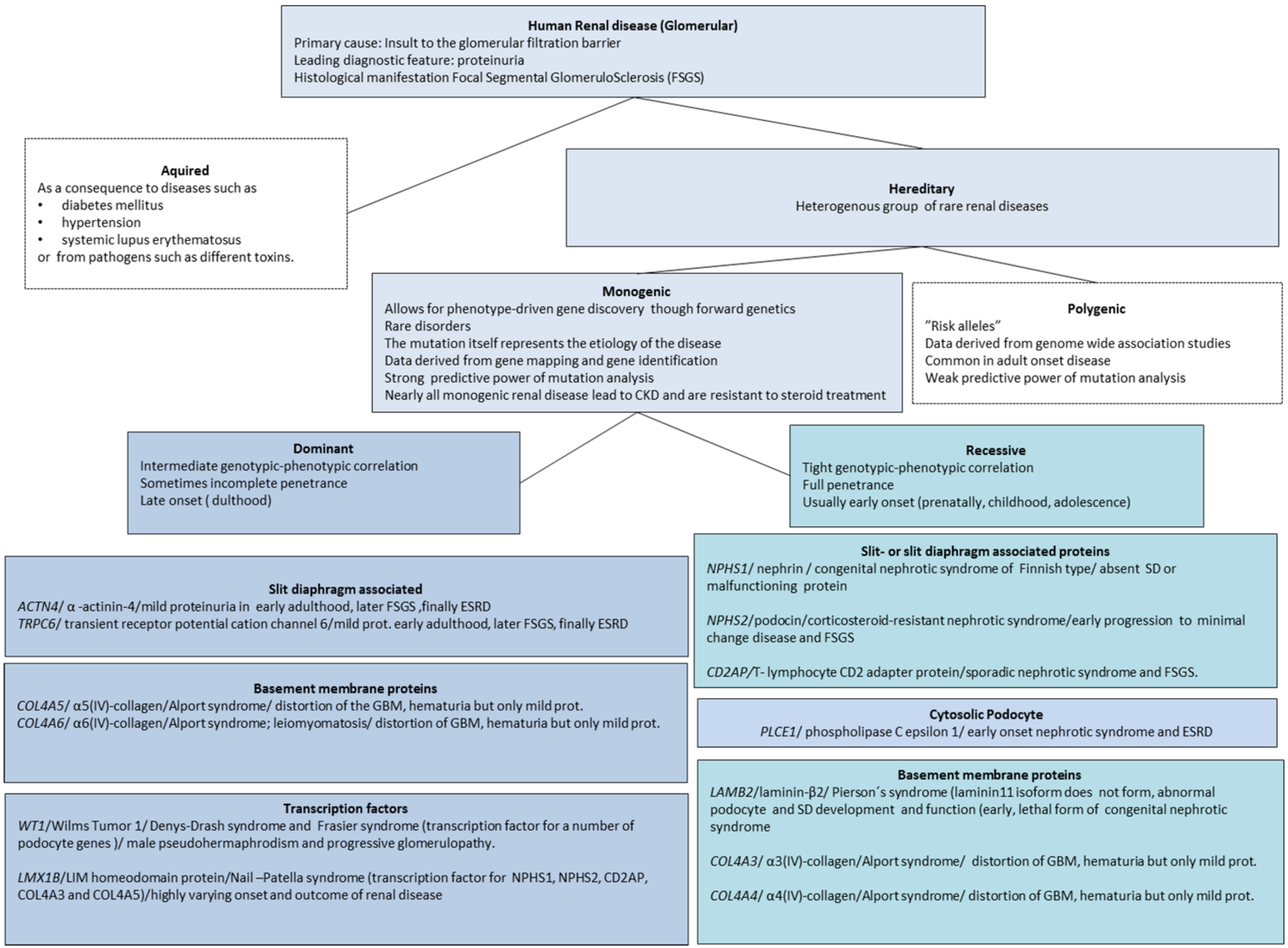
MAGI-2, member of the membrane associated guanylate kinase (MAGUK) family of scaffolding proteins is highly expressed in the synaptic junctions of mouse brain (217, 218) and glomerular podocytes (219). Through its PDZ, WW, and GUK domains it acts as a scaffolding protein coordinating signaling complexes (220-223) and has been identified as an interaction partner of nephrin, IQGAP and CASK in rat glomeruli (224). *Magi-2* null mice present with early-onset, progressive proteinuria and non-inflammatory, proliferative glomerulopathy with collapsing and crescent-like characteristics within a few weeks after birth. The pathological process coincides with decline of nephrin expression and the upregulation of CIN85 (225) which is a protein regulating the turnover of slit diaphragm complex proteins (226).

1.5.6 PLCε1

PLCε1 is a lipase cleaving phosphatidylinsositol-4,5-bisphosphate (PIP_2) to the second messengers inositol1,4,5-triphospahte (IP_3) and diacylglycerol (DAG) (227) and is thus involved in intracellular signaling pathways. It plays an important role in Ca^{2+} release both from internal stores as well as influx from cat ion channels such as TRCP6 (228). In the podocytes it is has been identified from the early S-shaped stage and is highly expressed in both cell body and foot processes at the late capillary stage (229). Even though PLCε1 null mice do not display a renal phenotype (230) positional cloning has identified PLCε1 in families with early onset nephrotic syndrome and ESRD, histopathologically displaying diffuse mesangial sclerosis and FSGS and histochemistry showing reduced nephrin expression (229). The importance of PLCε1 for the integrity of the filtration barrier has also been described in zebrafish where PLCε1 anti sense morpholino knock down and subsequent injection of a 500-kD Dextran labeled FITCH tracer molecule into the blood stream showed FITC-positive endocytosed vehicles in the epithelial cells of the pronephric tubule (229). PLCε1 is an exception to the general trend where mutated genes causing FSGS are most often structural components of the podocytes.

1.6 OVERVIEW GLOMERULOPATHY

Renal disease is not one disease but a generic term describing the inability of the kidneys for one reason or another to excrete wastes and maintain the electrolyte balance. Even when narrowing down the focus to the filtering unit itself, the glomerulus, now describing the disease as ”glomerulopathy”, the etiologies are diverse even if the disease commonly manifests as proteinuria. Most often “glomerulopathy” implies a non-inflammatory condition whereas the term “glomerulitis” is used when inflammation is involved. As the filtration barrier composes the integral part of the glomerulus, glomerulopathy often implies a malfunctioning interplay for one or another reason between the constituents of the GFB, and the disease can be primary due to gene mutations or pathogens, but it may also be secondary to other conditions such as hyperglycemia and hypertension. Genetic mutations leading up to primary disease can be monogenic, recessive or dominant, or they can be polygenic where the disease arises as a combined effect of mutations in two or more genes. As monogenic diseases, contrary to polygenic ones, have a tight genotypic-phenotypic relationship with almost always “full penetrance” (clinical symptoms are present in all individuals who have the disease-causing mutation) forward genetic studies of such human renal disease causing mutations giving rise to an observed phenotype has contributed greatly to our knowledge of GFB composition. Therefore, the presented chart (see page 28) that gives an overview of human glomerular disease causing mutations is focused on such monogenic diseases.


Human Renal disease (Glomerular)
Primary cause: Insult to the glomerular filtration barrier
Leading diagnostic feature: proteinuria
Histological manifestation Focal Segmental GlomeruloSclerosis (FSGS)
Aquired
As a consequence to diseases such as
• diabetes mellitus
• hypertension
• systemic lupus erythematosus
or from pathogens such as different toxins.
Hereditary
Heterogenous group of rare renal diseases
Monogenic
Allows for phenotype-driven gene discovery though forward genetics
Rare disorders
The mutation itself represents the etiology of the disease
Data derived from gene mapping and gene identification
Strong predictive power of mutation analysis
Nearly all monogenic renal disease lead to CKD and are resistant to steroid treatment
Polygenic
"Risk alleles"
Data derived from genome wide association studies
Common in adult onset disease
Weak predictive power of mutation analysis
Dominant
Intermediate genotypic-phenotypic correlation
Sometimes incomplete penetrance
Late onset (dulthood)
Recessive
Tight genotypic-phenotypic correlation
Full penetrance
Usually early onset (prenatally, childhood, adolescence)
Slit diaphragm associated
ACTN4/ α -actinin-4/mild proteinuria in early adulthood, later FSGS ,finally ESRD
TRPC6/ transient receptor potential cation channel 6/mild prot. early adulthood, later FSGS, finally ESRD
Basement membrane proteins
COL4A5/ α5(IV)-collagen/Alport syndrome/ distortion of the GBM, hematuria but only mild prot.
COL4A6/ α6(IV)-collagen/Alport syndrome; leiomyomatosis/ distortion of GBM, hematuria but only mild prot.
Transcription factors
WT1/Wilms Tumor 1/ Denys-Drash syndrome and Frasier syndrome (transcription factor for a number of podocyte genes)/ male pseudohermaphrodism and progressive glomerulopathy.
LMX1B/LIM homeodomain protein/Nail –Patella syndrome (transcription factor for NPHS1, NPHS2, CD2AP, COL4A3 and COL4A5)/highly varying onset and outcome of renal disease
Slit- or slit diaphragm associated proteins
NPHS1/ nephrin / congenital nephrotic syndrome of Finnish type/ absent SD or malfunctioning protein
NPHS2/podocin/corticosteroid-resistant nephrotic syndrome/early progression to minimal change disease and FSGS
CD2AP/T- lymphocyte CD2 adapter protein/sporadic nephrotic syndrome and FSGS.
Cytosolic Podocyte
PLCE1/ phospholipase C epsilon 1/ early onset nephrotic syndrome and ESRD
Basement membrane proteins
LAMB2/laminin-β2/ Pierson´s syndrome (laminin11 isoform does not form, abnormal podocyte and SD development and function (early, lethal form of congenital nephrotic syndrome
COL4A3/ α3(IV)-collagen/Alport syndrome/ distortion of GBM, hematuria but only mild prot.
COL4A4/ α4(IV)-collagen/Alport syndrome/ distortion of GBM, hematuria but only mild prot.

2 AIMS OF THE STUDY

The aims of this study were to increase current knowledge of the glomerular filtration barrier in health and disease by studying novel components, gene regulation and cellular aspects.

The specific objectives were:
Paper I: Examination of the expression and role of TDRD5 in podocytes with emphasis on the transcriptional and protein levels using in vivo as well as in vitro methods.

Paper II: Determination of the global expression signature of renal glomeruli during progression of Adriamycin-induced nephropathy in mice using RNA sequencing.

Paper III: Characterization of a novel podocyte specific protein Rhophilin1 and its function in the maintenance of the actin cytoskeleton in mouse podocytes.

Paper IV: Further studies on the effect of a single nucleotide polymorphism (SNP) r1866813, on differential expression of NCK1 the gene of which has been genetically associated with diabetic nephropathy.

3 EXPERIMENTAL PROCEDURES

The methods used in papers I-IV are described in detail in respective Material and Methods sections.

3.1 ANTIBODIES

Table 1. Primary antibodies used.

(Alexa Fluor secondary antibodies (Life Technologies) were used in addition).

Antibody	Product nr	Company	Study
β-actin	ab8227 (rabbit)	Abcam	[I,III, IV]
c-myc	M4439 (mouse)	Sigma-Aldrich	[III]
c-myc	C3956 (rabbit)	Sigma-Aldrich	[I, III]
dendrin	(64) (rabbit)		[III]
f-actin (rhodam.-phallod.)		Life Technologies	[III]
GFP	A11122 (rabbit)	Invitrogen	[III]
HIC5	611164 (mouse)	BD transd. laboratories	[III]
IL-20rb	ab95824 (rat)	Abcam	[IV]
NCK1	Ab32120 (rabbit)	Abcam	[IV]
NPHS1	BP5030 (guinea pig)	Acris GmbH	[III]
p-Myosin Light Chain 2	3675 (mouse)	Cell Signaling Techn.	[III]
NPHS2	p0372 (rabbit)	Sigma-Aldrich	[I]
RHPN1	sc-1940 (goat)	Santa Cruz	[III]
RHPN1	SAB1408228 (mouse)	Sigma-Aldrich	[III]
RHPN1	(208) (rabbit)		[III]
STAG1	#A300-157A(goat)	Santa Cruz	[IV]
SYNPO	sc-50459 (rabbit)	Santa Cruz	[III]
TDRD5	HPA014563 (rabbit)	Atlas Antibodies	[I]
TDRD5	HPA029418 (rabbit)	Atlas Antibodies	[I]

Table 1. Antibodies may be monoclonal or polyclonal.
Monoclonal antibodies bind to the same epitope as they are derived from identical immune cells.
Polyclonal antibodies are derived from several different immune cells and react with different epitopes. However, most often the host is immunized with a sequence of the target protein fused to a “tag”- sequence that allows for affinity purification of the produced antibodies giving rise to monospecific antibodies.

3.2 BLOOD UREA NITROGEN ANALYSIS (BUN) [II]

As a measurement of glomerular filtration rate and thus renal health the assay measures the level of the metabolic waste product urea. BUN was quantified using QuantiChrom Urea Assay Kit (BioAssay System), which uses a chromogenic reagent that forms a colored complex specifically with urea. The color intensity is directly proportional to the sample urea concentration. The assay was used as a quantification of glomerular disease progression in Adriamycin injected mice [II].

3.3 CONFOCAL MICROSCOPY [III]

Confocal microscopy is an imaging technique allowing for increased optical resolution and contrast of a micrograph by the addition of a pin-hole at the confocal plane of the lens to eliminate out-of-focus light. A reconstructed three-dimensional image is then assembled from the sampled images. The technique was used in III to image immuno-fluorescent cells and kidney samples.

3.4 CREATININE ASSAY (SERUM/ URINE) [II, III]

Creatinine is synthesizes from creatine, which is produced during muscle contraction at a fairly constant rate. Creatinine is removed from the blood by filtration in the glomerulus and excreted in the urine. When glomerular filtration rate is lowered due to kidney disease blood creatinine levels are increased, and urinary levels are diminished. Serum creatinine levels were measured (QuantiChrom™ Creatinine Assay Kit (BioAssay Systems, Hayward, CA) using picrate that forms a red colored complex with creatinine which was subsequently quantified. The assay was used in papers II and III to evaluate urine and serum creatinine levels respectively in mice.

3.5 ELECTRON MICROSCOPY (SCANNING (SEM) / TRANSMISSION (TEM)) [I, II, III]

An imaging technique that uses scattered (SEM) and transmitted (TEM) electrons respectively as a source of illumination. They differ in application as SEM focuses on the sample's surface creating a 3D-image whereas TEM provides the details about internal composition requiring thin sections of the sample. As the electron wavelength is 100,000 times shorter than that of photons, it has a higher resolving power than light microscope. TEM was used in papers I, II, and III to ultrastructurally determine the morphology of sectioned zebrafish [I], and mouse glomeruli [II, III], whereas SEM was used in III to visualize the surface of the mouse glomerular tuft.

3.6 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) [II, III]

Enzyme-linked immunosorbent assay (ELISA) is a biochemical method used to detect and quantify substances such as albumin in biological samples. In an ELISA, the antigen of interest is immobilized to a solid surface and then complexed with an enzyme-linked antibody. Detection is accomplished by incubating with a substrate that the enzyme will convert to some detectable signal, most commonly a color change that may be quantified. A so called competitive version (Albuwell, Exocell) of the method was used in II and III to quantify urinary albumin where albumin of the stationary phase and albumin of the urine sample were competing in the binding to added anti-albumin antibodies. After washing only the albumin bound antibody that was bound to the stationary phase remained in the well. Detection was accomplished by the addition of anti- IgG -HRP conjugate and a chromogenic substrate that was oxidized by the horse radish peroxidase (HRP) producing a color of a quantifiable intensity.

3.7 ETHICS STATEMENT [I, II, III, IV]

All the experimental studies presented were conducted according to regulations related to handling of laboratory animals and approved by local ethical committees.

3.8 FLUORESCENCE-ACTIVATED CELL SORTING (FACS) [I, III]

A method for sorting a heterogeneous mixture of cells based on the light scattering or fluorescent characteristics of the cells. In brief, the cells are suspended in a stream of fluid whereby they are passed through a fluorescence measuring station after which a charge is applied to the molecules based on their fluorescence intensity. The charged droplets are then fractionated based on their charge. The method was used in papers I and III where total glomerular cell populations from bi-transgenic mice were fractionated.

3.9 GLOMERULI ISOLATION [I, II, III, IV]

A technique developed at Göteborg University by Dr Minoru Takemoto (231) where glomeruli is isolated from mouse kidney by perfusion though the heart using spherical super-paramagnetic Dynabeads (Ø 4.5 μm). The magnetic beads get stuck in the glomeruli which can be collected using a magnet. The large amount of RNA subsequently extracted from such glomeruli was a prerequisite for papers I, II, III and IV.

3.10 HISTOCHEMISTRY [I, II, III]

This is a technique that uses biochemistry to visualize the morphology of cells and tissue.

3.10.1 Hematoxylin/ eosin staining (HE) [I, II]

Hematoxylin stains nucleic acids (deep-purple) whereas eosin stains proteins (pink) nonspecifically. The method was used in I to investigate the morphology of the zebrafish pronephros in *tdrd5* mutants. In II it was used to investigate the morphology of kidney sections of Adriamycin-injected nephrotic mice.

3.10.2 Periodic acid-Schiff staining (PAS) [III]

Periodic acid–Schiff staining is a method that is used to detect carbohydrates such as polysaccharides, glycoproteins and glycolipids and is therefore often used to stain structures containing a high proportion of these macromolecules such as connective tissue and basal lamina. The periodic acid oxidizes diols of the sugar into aldehydes. The method was used in paper III to visualize the morphology of mouse kidney tissue from *Rhpn1*-/- mice.

3.11 IMMORTALIZED HUMAN PODOCYTE CELL CULTURE [III]

Terminally differentiated podocytes do not maintain their unique phenotype in cell cultures. However, it is possible to extensively maintain the podocyte properties in cell culture using an immortalized podocyte line. A human immortalized podocyte cell line used in III was generated by transfection with the temperature-sensitive proto-oncogene SV40-T (232) which makes the cell to proliferate at the permissive temperature 33 ^{0}C. The SV40-T gene is then turned off after moving the cells to the non-permissive 37 ^{0}C whereby they enter growth arrest and take on more podocyte characteristics. In III we used the cell line to investigate RHPN1 effect on the podocyte actin cytoskeleton.

3.12 IMMUNOFLUORESCENCE STAINING [III, IV]

Both direct and indirect immunofluorescence staining methods were used in III and IV. DAPI (4', 6-diamidino-2-phenylindole) which is a fluorescent stain that binds A-T rich regions in DNA was used to image nucleus in a direct way. Phallodin conjugated to the red-orange fluorescent dye tetramethylrhodamine was used to image filamentous actin. Other proteins of interest were imaged indirectly by incubation with IgG-conjugated fluorophores (Alexa Fluor Dyes, Life Technologies) which targeted protein specific primary antibodies. The technique was used on cells [III] and on kidney section [III, IV].

3.13 IMMUNOGOLD ELECRON MICROSCOPY [I]

A staining technique used with transmission electron microscopy where colloidal gold is attached to antibodies designed to cross-react with specific proteins. The technique was used in paper I where TDRD5 was found to be localized to podocytes in human kidney sections.

3.14 IN SITU HYBRIDIZATION [I]

In situ hybridization is used for localization of mRNA transcripts in situ using its corresponding antisense sequence as a labeled RNA probe. The method was used in I where Digoxigenin (DIG) conjugated to uridine (Roche Applied Science) was incorporated into RNA using RNA polymerase. The riboprobe then hybridizes to the sense mRNA on snap frozen mouse kidney sections facilitating its detection using a DIG-antibody conjugated to alkaline phosphatase whereby it was visualized using NBT and BCIP.

3.15 LUCIFERASE ASSAY [IV]

The Dual-Luciferase® Reporter Assay System (Promega) allows for the quantification of the ability with which a sub cloned sequence activates the firefly (*Photinus pyralis*) luciferase reporter sequence. A vector carrying the Renilla (*Renilla reniformis)* luciferase reporter gene is co transfected acting as an internal control. The system was used in IV as a mean of investigating the ability of the different *cis*-acting SNP at the 3q locus to activate the expression of NCK1.

3.16 MOUSE GENE TARGETING TECHNIQUE [III]

A reverse genetic technique that awarded Mario R. Capecchi, Sir Martin J. Evans and Oliver Smithies The Nobel Prize in Physiology or Medicine 2007. The method is based on the transfer of a DNA sequence which is homologous to the endogenous mouse gene to be targeted except for a desired mutation and with the addition of a selection cassette into stem cells isolated from a mouse blastocyst (white mouse). Through homologous recombination the introduced sequence will occasionally be incorporated into the genome. Cells are grown in selective media and cells in which incorporation of the introduced sequence has taken place will be able to expand due to resistance conferred by their selection cassette. Such cells are analyzed using Southern Blot to confirm that the recombination has occurred in the desired location. Clones of correctly targeted cells are then introduced into a blastocyst (black mouse) and implanted into a pseudo pregnant female. The resulting pups will be chimeras (black/white). In some of the chimera the mutation may be transmitted to the germ cells producing sperm and eggs with the mutated gene. When such chimeras are crossbred with wild type mice some of the offspring will have one copy of the knocked-out gene in all their cells (white mice), but they are still heterozygous. Interbreeding of these heterozygous offspring will produce some mice that are homozygotes of the mutation. The technique was used to produce an *Rhpn1* knock out mouse [III] in which exons 1-4 of the *Rhpn1* gene were substituted with a Green Fluorescent Protein (GFP) cassette.

3.17 PHASE CONTRAST MICROSCOPY [III]

An optical microscopy technique that converts the phase shifts of light passing through a transparent media into brightness changes of the image. The microscope was used in III to visualize isolated glomeruli.

3.18 PLASMIDS AND CLONING [I, III, IV]

A plasmid is a small circular DNA molecule that within biotechnology is used for the cloning and amplification of DNA sequences through the transformation (into bacteria) or transfection (into mammalian cells) whereby the endogenous replication machinery is used for the amplification into a desired amount of copies.
The technique was used in I to amplify both *Tdrd5* full length transcripts cloned into the xhoI and sfiI restriction sites of pCMV-Myc vector (Clontech). The riboprobe used in I was produced from a 598-bp *Tdrd5* cDNA fragment cloned into pCRII-TOPO Dual Promoter Vector (Invitrogen). In III the technique was used when human RHPN1 was expressed in mammalian cells using the pcDNA4/TO/myc-His-A expression vector (Invitrogen). The construct was prepared by PCR amplification of the full-length cDNA encoding human *RHPN1* from a corresponding IMAGE cDNA clone IRATp970F0343D and subsequently cloned into the EcoR1-XbaI sites of pcDNA4/TO/myc-His-A. In order to evaluate the *cis*-regulatory potentials of three human conserved sequences with single nucleotide polymorphisms in IV both in vitro and in vivo DNA sequences were amplified using human material and cloned into pCRII-TOPO vectors (Invitrogen) and then subsequently sub cloned into either the luciferase pGL4.26 plasmid (Promega) between SacI and XhoI sites or a Tol2-based plasmid containing a GFP cassette under the *Nphs2* promoter.

3.19 POLYMERASE CHAIN REACTION (PCR) [I, III]

A biochemical method in which cDNA is amplified through a series of annealing/ elongation cycles. The method was used in I to determine *Tdrd5* expression in a panel of mouse tissues using a commercial cDNA library (Clontech Mouse Multiple tissue cDNA MTC panel 1) and in III to amplify full-length human *RHPN1* from a corresponding IMAGE cDNA clone IRATp970F0343D.

3.20 PRIMARY CELL CULTURE [III]

We used primary podocyte cell cultures from seeded mouse glomerular explants of both wild type and *Rhpn1* knock out animals [III] in order to asses RHPN1 subcellular expression pattern and as an attempt to delineate its molecular function.

3.21 PROTEIN INTERACTION NETWORK ANALYSIS [II]

The protein–protein interaction information was downloaded from the Human Protein Reference Database (http://www.hprd.org/, release 9). The database curated the human protein-protein interaction information from published literatures. The mouse genes were mapped to the corresponding homologous human genes by using the NCBI HomoloGene assembly (http://www.ncbi.nlm.nih.gov/homologene). The R program (http://www.r-project.org/) was used for the data processing, and Rgraphviz package was used to visualize the interaction networks (233).

3.22 QUANTITATIVE REAL TIME –PCR (QRT-PCR) [I, III, IV]

A method based on the same principle as the polymerase chain reaction but with the main difference that the amplified DNA is detected as the reaction progresses (in real time (RT-)) making it a quantitative (q-) method. The method was used in I to quantitatively determine *tdrd5* expression in fractioned zebrafish tissue from wt as well as from *tdrd5* mutant larvae. In III and IV the method was used to quantitatively determine *Rhpn1, Rhpn2, Synpo* [III] and *Nck1* [IV] expression in mouse glomeruli as well as in differentiated/undifferentiated immortalized human podocytes [III].

3.23 REVERESE TRANSCRIPTION [I, III, IV]

Reverse transcription is a method where the enzyme reverse transcriptase is used to generate a complementary DNA (cDNA) sequence from a RNA template. It was used to generate cDNA from RNA isolated from mouse glomeruli [I, III, IV] and from zebrafish glomeruli and fractionated zebrafish larvae homogenate [I]. In IV the method was used generate cDNA from transformed primary human lymphocytes.

3.24 REVERSE TRANSCRIPTION-PCR (RT-PCR) [I, III]

A biochemical method where cDNA reversibly transcribed from isolated RNA using the reverse transcriptase enzyme is amplified through a series of annealing/ elongation cycles. In I the method was used to qualitatively determine *Tdrd5* transcript localization to fractioned mouse kidney tissues samples (glomeruli and kidney fraction lacking glomeruli), as well as for amplification of both *Tdrd5* transcripts for subsequent cloning purposes. In I and III it was used to qualitatively determine transcript expression in FAC sorted total mouse glomerular cell-population.

3.25 RNA SEQUENCING [II]

In II the RNA library construction and RNAseq were performed at BGI-Hong Kong Co., HKG using the Illumina HiSeq 2000 platform and True Seq library construction (Illumina).

3.26 SDS-PAGE [II, III]

A biochemical method that utilizes the anionic detergent Sodium dodecyl sulfate in SDS- polyacrylamide gels to impart a negative charge to proteins in this way linearizing and adding a negative charge to them which makes it possible to separate them during electrophoreses. The gel may then be used further for Western Blotting [I, III, IV] or as in paper II and III where the gel was stained with Coomassie Brilliant Blue to analyze the existence of albumin in urinary samples from mice.

3.27 STATISTICS [I, II, III, IV]

Data of luciferase assay [IV] and qPCR in I, III, IV was statistically analyzed by *t*-test. In II statistics test were performed using the global test algorithms in the Cufflinks package (234) in order to identify the genes differentially expressed between the different stages of ADR treated glomerular samples and the control.
In IV the Fisher's exact test was used for analysis of zebrafish GFP expression rate. The Fisher's exact test and *t*-test were used for association analysis of clinical data with genotypes [IV].

3.28 TRANSGENIC ANIMALS [I, III, IV]

The definition of a transgenic animal is when genetic material has been transferred naturally, or by genetic engineering from one organism to another. In III a *Rhpn1* null mouse was created according to the gene targeting technique described previously. The excised exons 1-4 of *Rhpn1* were here substituted for a green fluorescent protein cassette. In III a mouse expressing enhanced yellow fluorescent protein (EYFP) in the podocytes was produced by crossing a mouse expressing the EYFP gene under the Gt(ROSA)26Sor locus with an interposed floxed STOP sequence with a transgenic mouse expressing the Cre recombinase under the control of the *Nphs2* promoter. FAC sorted glomerular cells from this mouse strain was also used in I. Rhophilin-1/Rhophilin-2 double knock out mice were generated by intercrossing *Rhpn1*$^{+/-}$ and *Rhpn2*$^{+/-}$ mice of a mixed C57BL/6, 129Sv, and CD1 genetic background III. *RhoA* podocyte-specific deletion in mouse of a Rhophilin-1 null background was achieved by crossing triple transgenic heterozygous mice (*Rhpn1*$^{+/-}$**;** *Nphs2*$^{cre/+}$**;** *RhoA*$^{fl/+}$) of a mixed C57BL/6 and 129Sv genetic background [III]. A transgenic zebrafish line (235) was used in I that express GFP under the podocyte specific podocin promoter allowing for the visualization of the pronephric glomerulus.

3.29 TRANSIENT TRANSFECTION [I, III, IV]

Transfection is the process of introducing nucleic acids into cultured metazoan cells by opening transient pores in the cell membrane allowing uptake of material. The transient transfections were performed using Lipofectamine 2000 (Invitrogen) which is a cationic lipid that encloses the nucleic acid forming liposomes that fuses with the cell membrane and deposit their cargo inside the cell. The method was used in paper I to introduce expression vectors containing the two myc-tagged *Tdrd5* splice variants into human embryonic kidney (HEK) cells in order to produce myc-tagged proteins by using the cells protein expression system. In III we transiently transfected and expressed RHPN1 and a constitutively active RHOA construct to investigate their separate and concerted role on a cellular level. In paper IV HEK cells were transfected with expression vectors containing various single nucleotide polymorphism sequences cloned in frame with a luciferase cassette were after the expressed products were used in the luciferase assay.

3.30 TUNEL STAINING [II]

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is a biochemical method that detects DNA fragmentation that is result from apoptosis.

The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes the addition of the secondarily labeled dUTP to the nicked DNA, thus visualizing the break. TUNEL staining was used in paper II to visualize apoptosis in glomeruli of Adriamycin injected mice.

3.31 WESTERN BLOT [I, III, IV]

An analytical technique used to detect specific proteins in a sample of tissue homogenate or extract which is separated by electrophoreses on a gel whereby the proteins are blotted on to a membrane, with the subsequent incubation with protein- specific labelled antibodies. Detection is achieved by incubation with a HRP conjugated secondary antibody raised against IgG from the host of the primary antibody. The HRP, in the presence of a chromogenic substrate, produces a detectable signal.

3.32 ZEBRAFISH MORPHOLINO KNOCK DOWN TECHNIQUE [I]

The method is used as a reverse genetic tool where genes are repressed using antisense morpholinos whereby the phenotype is analyzed. Morpholinos are modified ~25-base long antisense nucleotides that are injected into the yolk of fertilized zebrafish egg at the 1-2 cell stage. The morpholino then diffuses from the yolk into the dividing cell/cells. The antisense oligo could be designed to hybridizes to a pre-mRNA at a splice site hindering the correct splicing of the transcript (splice blocker) or it could be designed to hybridize to the ATG site blocking its translation on the ribosomes (translational blocker). At 4 dpf the phenotype of the larvae is analyzed. The technique was used in paper I where *tdrd5* was repressed using both a translational blocker and a splice blocker.

4 RESULTS AND DICUSSION

The kidneys have several function one of them being to filter waste products from the blood and excrete them through the urine. The ultimate structure responsible for this sieving is the glomerular filtration barrier containing mesangial and endothelial cells as well as podocytes, together with the glomerular basement membrane and the slit diaphragm. Proper structure, function and concerted cellular interplay are a prerequisite for renal health. Glomerular damage underlies as much as two-thirds of all chronic kidney disease cases leading to ESRD that constitutes an enormous socioeconomic burden. The molecular pathomechanism are likely to be many and the causes of most glomerular diseases are poorly understood. The present work is a part of a large project aimed at generating new knowledge about glomerular development, function and disease. Papers I and II are the direct result from the large-scale initiative (236-238) where RNA from normal and healthy glomeruli were used to assemble a glomerulus specific GlomChip from which both *Tdrd5* and *Rhpn1* were identified. For paper III the large amount of glomeruli and subsequent RNA isolation was imperative for the state of the art RNA deep sequencing undertaken. Isolation of glomeruli from two mouse strain carrying the nephropathy-prone 129/Sv allele and nephropathy-resistant C57BL/6 allele respectively identified NCK1 as a protein differentially expressed in these mouse strains.

4.1 PAPER I

Our previous efforts to profile the global transcriptome of the kidney glomerulus through microarrays (238) revealed *Tdrd5* as a highly enriched transcript. *Tdrd5* draw our attention as it was positioned head to head with the *Nphs2* gene which is a podocyte-specific protein associated with hereditary proteinuric disease (175), a genomic arrangement that was shown to be conserved. Consequently, it was decided to further investigate and characterize TDRD5's role.

In order to investigate TDRD5 localization at the transcriptional level I used RT-PCR on mouse cDNA tissue panel library, FAC sorted mouse podocytes and mouse glomeruli as well as in situ hybridisation on mouse snap frozen kidney sections, whereby I show that *Tdrd5* is an, apart from mouse testis (239, 240), a podocyte specific transcript with two splice variants. Furthermore it was shown by qPCR on cDNA reversibly transcribed from RNA isolated from GFP-expressing glomeruli of 3dpf transgenic zebrafish larvae (235), *tdrd5* to be glomeruli enriched transcript. TDRD5 protein localized to mouse glomeruli as determined by Western Blot using Myc-tagged transcripts of the two splice variants as positive controls. Immuno Gold Electron Microscopy on human kidney sections further specified the protein expression to be enriched in the foot-processes of the podocytes. Morpholino injection using both translational and transcriptional blockers into fertilized zebrafish egg at 1-2 cell stage was used to study *tdrd5* ablation in an in vivo setting. The histological effects of *tdrd5* knock down were studied using hematoxylin/eosin staining on 6 µm zebrafish sections which revealed a disturbed glomerular morphology and distended tubules. *Tdrd5* knock down effect on the filtration barrier ultra-structure depicted by transmission electron microscopy revealed grossly unaffected podocytes but with a disturbed capillary endothelium and distended capillaries, which were also reduced in number.

The results of this paper positioned the *TDRD5* head-to-head with the disease-linked *NPHS2* gene (175) in vertebrate genomes and revealed highly conserved sequences through different species. Very little is known about TDRD5, but it has previously been shown to be highly expressed in the mouse testis (239, 240). Our studies demonstrated that *TDRD5* is also highly expressed in renal podocytes, importantly; the protein is likely to have a role in glomerular development and function as silencing of *tdrd5* expression in zebrafish resulted in pronephros abnormalities. As yet, we or others have not yet shown the gene to be associated with any human kidney disease.

4.2 PAPER II

One way to increase the understanding of mechanisms for glomerular disease is to elucidate glomerular expression signatures using either microarrays or RNAseq. This needs to be done for many glomerulopathies and glomerular disease models to obtain a good picture of the pathogenic processes in the kidney.

In this study, we made use of an Adriamycin-induced FSGS-like (focal segmental glomerular sclerosis-like) proteinuric mouse model. In this model, severe proteinuria develops, reaching maximum at 7 days after injection of Adriamycin. Then there is partial recovery, but the glomerular function and pathology do not normalize. The mice were weighed on a daily basis and spot-urine, blood samples, kidneys as well as RNA extracted from glomeruli were sampled during the 14 day duration of the experiment.

Weight curves, urinary albumin and urinary albumin to creatinine levels as well as blood urea nitrogen levels all witness of an acute phase of renal injury day4 reaching its maximum at day7 where after urinary and blood levels are somewhat alleviated. Ultrastructurally segmental foot processes effacement was evident at day seven and at 14 days histology revealed resorption droplets and intraluminal casts in the tubular compartment with some glomeruli showing segmental sclerosis.

The acute phase of injury may be related to the effect of Adriamycin which is known to induce DNA damage through reactive oxygen species (ROS) (241) something that seem to be reflected in the p53 pathway being the most significantly upregulated pathway day4 according to the RNA-seq data, with Psrc1 and Eda2 being the top up-regulated genes. They are both transcriptional target of p53 (242, 243). Even though p53 is not itself differentially regulated in our screen, Mdm2 which is a protein that functions to keep p53 at low levels in unstressed cells by continuous ubiquitination targeting p53 for degradation (244), is in our screen significantly regulated day4. The protein-protein interaction network described previously (245) linked Mdm2 to the expression of Trp53inp1, Hipk2, Pja1, Rpl11 and Ccng which are all significantly regulated, as well as Sesn2 and Gpx1 two antioxidant genes that p53 trans activates to keep ROS at non-toxic levels at physiological conditions (246). The TUNEL staining also reflect the apoptosis of glomerular cells as the number of apoptotic positive cells gradually increased after ADR injection, reaching maximum at 14 days after ADR injection.

The Adriamycin mouse model is characterized by podocyte foot process effacement which means there is a high turnover of cytoskeletal components or molecules related to cytoskeleton maintenance. As such activities are known to induce ER stress (247), the differential regulation of Hspa5, Caspase12 and Ddit3, three molecules that are components of the ER stress pathway in podocytes (248), could be expected. Collectively, these observations suggest that podocyte apoptosis during progression of glomerulopathy might be associated with ER stress.

In this study, as we have done in two previous unpublished global glomeruli transcript screens, one of them being data derived from RNA-sequencing of *Rhpn1* null mice found the hemoglobin genes, Hba-a1 and Hbb-bs to be the two most significantly down regulated transcripts day4. We find it likely that the expression of Hbb-bs and Hba-a1 in our screen stems from the mesangial cell population of the glomeruli as a previous report has localized the hemoglobins to the rat glomerular mesangial cells both on transcriptional as well as on protein level (249). In the same study the authors show that the hemoglobins are transiently up regulated under the prevailing experimental hypoxic conditions (249). The investigators also overexpressed the α and β-globins in the SV40-MES13 murine mesangial cell line and found that reactive oxygen species production was reduced thus ameliorating oxidative stress when induced by hydrogen peroxide. Based on their results they speculate that the proteins potential function in the mesangial cells includes antioxidant defense. Thus, we speculate that the regulation of these transcripts may be related to a cellular response acting on oxidative stress. The globin family today consists of the hemo- and myoglobin (250), plus the more recently discovered ubiquitously expressed cytoglobin (251) and neuroglobin which is predominately expressed in the nerve cells (252). They are all found to bind oxygen in a reversible way but the cytoglobin and neuroglobin are furthermore believed to act as O_2 consuming enzymes or as O_2 sensors or might being involved in NO/O_2 chemistry (253). Interestingly neuroglobin was also found to be significantly down regulated day 4 and 7 in our screen. Neuroglobin is mainly found in the nervous system and considering the close relationship between the transcriptome of the neurons and the podocytes (23) we find it likely that the neuroglobin expression is of podocyte origin.

The circadian rhythm pathway was the highest regulated pathway between control and day 7, with 27% of the member proteins being differentially regulated. That the renal function is oscillating according to circadian rhythmicity pattern was first documented in the middle of 19th century (254). The proteins constituting the core of the feedback loop that generates the transcriptional rhythms of approximately 24 h periodicity BMAL1, CLOCK and NPAS2 which dimerize into various heterodimers whereby they activate the transcription and translation of their repressors PER and CRY (255) during the daytime, are down regulated in our screen, all of them except CLOCK significantly. Moreover the melatonin receptor Mt2 which is a G-protein coupled, 7-transmembrane receptor responsible for melatonin effects on mammalian circadian rhythm was significantly down regulated day4 and 7. Considering the large fraction of the regulated proteins involved in the circadian rhythm and the melatonin receptor it was not very surprising to find all three proteins taking part of the G protein cascade of visual transduction (256) Rhodopsin (Rho), Transducin (Gnat1) and cGMP phosphodiesterase ((Pde4b and 4d) (257)) to be significantly down regulated day4 and 7 (Rho, Gnat1 and Pde4d also day 14).

In order to identify the genes differentially expressed between the different stages of ADR treated glomerular samples and the controls, statistics test were performed using the global test algorithms in the Cufflinks package (234), The result showed that 721 genes out of the 38,742 total genes detected were significantly differentially expressed between ADR-treated glomeruli at the 4-day stage and the controls (False Discovery Rate < 0.05), and 363 genes were differentially expressed genes at 7 days stage and 637 differentially expressed genes at 14 days stage.

We conclude that The ADR-proteinuric mouse model is a stable model of chronic progressive nephropathy, and that the transcriptome profile generated from RNAseq technology is valuable for dissecting the molecular mechanisms of the proteinuria.

4.3 PAPER III

RHPN1 was disclosed as one of the highest expressed glomerulus-enriched transcripts on the microarray previously performed by our group (238). Furthermore, the transcript was in the same study localized to the podocytes using in situ hybridization (238). Rhophilin-1 was identified almost 20 years ago as a Rho GTPase interacting protein (258) and in another study shown to be expressed in the fibrous sheet of the mouse sperm tail (259). To our knowledge, no data have since been presented when it comes to expression or biological function of this protein.

The aim of this paper was to further delineate RHPN1 spatiotemporal expression in glomeruli, as well as investigate by which mechanisms it exerts its functions using in vivo and in vitro methods.

We used frozen mouse kidney sections and FAC sorted mouse podocytes to show that *Rhpn1* is exclusively expressed in the podocytes of the late capillary stage of glomerulogenesis. We produced a *Rhpn1* -/- mouse where new-born pups were phenotypically normal at birth but developed albuminuria at two weeks of age. The glomeruli of these mice presented focal glomerular sclerotic lesions with an expanded mesangium similar to what may be seen in FSGS or diabetic nephropathy. Ultrastructurally, a widened glomerular basement membrane and effaced foot processes were noted indicative of dysregulation of the actin cytoskeleton. To further investigate the mechanism giving rise to the above phenotype we made use of primary podocyte cell cultures emanating from glomeruli of *Rhpn1* knock out and wild type mice, as well as immortalized human podocyte cell cultures. The primary podocytes showed RHPN1 to be expressed at the leading edge of the cell. Whereas the wild type podocytes showed a polarized and highly arborized subcortical actin cytoskeleton structure, the *Rhpn1 -/-* podocytes appeared more stellate and less curved at the plasma membrane with prominent ventral stress fibres spanning the cell body suggesting that RHPN1 interacts with cytoskeleton proteins. *RHPN1* transient transfection of immortalized human podocyte cells led to a substantial downregulation of the otherwise prominent actin stress fibres of this cell culture system. Transfection of a constitutively active RHOA expressing construct led to an expected increase of stress fibre formation, which we by co-transfection with *RHPN1* were able to alleviate. RHOA is acknowledged to be a major determinant for the formation of actomyosin stress fibre formation (260), and the stress fibre associated non muscle II regulatory light chain is known to be a target of ROCK which is and RHOA downstream effector (261). To this end we investigated the phosphorylation status of the stress fibre associated NM II RLC both in vivo in kidney sections of *Rhpn1*-/- mice and in *RHPN1* transfected / vector transfected immortalized human podocytes. These in vivo and in vitro studies both showed a marked increase of phosphorylated NM II RLC expression in the absence of *RHPN1*, indicating that RHPN1 is a regulator of RHOA activity. The actin cytoskeleton is appreciated to be a major constituent of podocyte physiology and function and RHOA together with RAC1 and CDC42 are well recognized as being the determinants of its proper regulation (209, 210, 262-264).

In summary, this study has identified Rhophilin-1 as a novel podocyte specific protein, regulating RHOA activity and the consequent phosphorylation of the non-muscle myosin regulatory light chain through RHOA downstream effectors, thus being an integral component of the glomerular filtration barrier. Its role in glomerular diseases remains to be studied.

4.4 PAPER IV

This paper is a part of studies in the laboratory on the potential regulatory role of a previously reported genetic association of the single nucleotide polymorphism (SNP), rs1866813 at 3q locus with diabetic nephropathy (265). The glomerulus is a primary target of this devastating diabetes complication, but the molecular pathomechanism of this disease is still poorly understood. The SNP is located approximately 70 kb downstream of a cluster of the four genes: STAG1, TMEM22, NCK1 and IL-20RB, of which mouse NCK1 has been associated with foot process formation during podocyte development in mice and regeneration following glomerular injury (266).

In this study we tested in a series of experiments whether the different allelic genotypes of the SNP differentially regulate downstream targets. In vitro, two alleles of the SNP show differential effects on luciferase activity in transfected cells. In vivo using transgenic zebrafish larvae we further demonstrate that two alleles of the SNP differentially regulate GFP expression in zebrafish podocytes. By immunofluorescence staining and Western blotting we showed that only NCK1 of the four genes was mainly expressed in mouse glomeruli where it was observed in podocytes. We also showed that genotypes of the SNP rs1866813 correlated with NCK1 expression in immortalized lymphocytes from diabetic patients, where the risk allele was associated with increased NCK1 expression compared to the non-risk allele. We also showed that in glomeruli *NCK1* is differential expressed in two mouse strains carrying the nephropathy-prone 129/Sv allele and nephropathy-resistant C57BL/6 allele respectively.

We conclude from this work that the DN-associated SNP rs1866813 is a remote *cis*-acting variant differentially regulating glomerular *NCK1* expression, and that glomerular NCK1 may play a role in the pathogenesis of diabetic nephropathy.

5 GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Since the first description of the slit diaphragm as imaged by Rodewald and Karnovsky in 1974 (140) using electron microscopy on mouse and rat kidney tissue, and the discovery of nephrin (153) and its subsequent localization to the slit diaphragm much has happened in the glomerular research area. Previously and historically the main filtering function of the GFB was attributed to the GBM but in the recent decades the research community has emphasize the podocytes as the major culprit of glomerular disease. Even though foot process effacement with the concomitant disappearance of the SD is noted in most cases when proteinuria is present, it is today recognized that the concerted interplay with all the GFB constituents is important for proper filtering function.

Considering the podocyte cell morphology and its transcriptome that closely resembles that of the neurons it is tempting to hypothesize around TDRD5 function in the podocytes. That the neurons are in need of the neuronal granules that tether mRNA at distant synapses for rapid translation is known and it is easy to draw parallels to the podocytes and the slit diaphragm. There is yet no published data of the existence of such granules but there is evidence that supports their existence. Considering TDRD5 function in the mouse germinal granules where it takes part in the scaffolding and processing of pi-RNA TDRD5 could be hypothesized to take part of such granules.

The development of RNA sequencing allows for high resolution identification of de novo transcripts in biological samples. The method is highly quantitative and currently even allows the generation of full transcriptomes from single cells (267). This opens up for extensive characterization of global expression signatures even from single glomeruli and the method is likely to yield a large body of exciting expression profiles during development and in disease in the near future. Such profiles can hopefully provide a new source for drug target discovery. The glomerular screen presented here on glomeruli in Adriamycin-induced nephrosis is an example which opens up for investigation of proteins and pathways that are regulated when podocytes are targeted. For example, the unexpected result that the hemoglobin alpha and beta chains being the most down regulated transcripts day4 as compared to control needs to be further investigated. ‘

The significance of RHPN1 regulation of RHOA and its effect on podocyte actin cytoskeleton is easy to appreciate as most mutations or injury mechanisms finally result in rearrangement of actin fibers, podocyte foot process effacement, and proteinuria. Thus further investigations need to be done when it comes to the fine-tuning of this relationship.

The finding presented in paper IV where the expression of NCK1 is shown to be regulated by the diabetic nephropathy associated allelic genotypes of SNP r1866813 highlights the level of complexity at which the genome is regulated. With the advent of new generation sequencing techniques the speed with which such intergenic regulatory elements are discovered will most certainly increase.

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